THE EFFECT OF GENDER ON THE RATE OF METABOLISM OF MIDAZOLAM IN HUMANS USING LIVER MICROSOMES

1997

HINKLE

THE EFFECT OF GENDER ON THE RATE OF METABOLISM OF MIDAZOLAM IN HUMANS USING LIVER MICROSOMES

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ABSTRACT

Midazolam (MDZ) is a short-acting benzodiazepine used in anesthesia for its anxiolytic, sedative, and amnestic properties. MDZ has a variable and unpredictable effect among individuals, which has the potential to complicate patient care and prolong hospital stay. It has been reported that inter-individual differences in the rate of metabolism are the primary cause of variances in the drug's therapeutic effect. MDZ is metabolized almost exclusively by the cytochrome P-450 3A subfamily (CYP3A). The drug is eliminated to a major metabolite, 1'-hydroxymidazolam, which accounts for at least 70% of an administered dose. MDZ forms a minor metabolite, 4-hydroxymidazolam, which comprises about 4% of an administered dose, and is also metabolized by CYP3A. The objective of this study is to determine, using human liver microsomes, if there is a gender difference in the rate of MDZ metabolism to both 1' and 4-hydroxymidazolam. To evaluate gender differences, microsomes from five male and seven female liver donors, unsuitable for transplantation, were chosen. Metabolic capacity of females, expressed as intrinsic clearance to degrade MDZ to 1'-hydroxymidazolam, is not significantly higher than males ($p < 0.06$). But, when postmenopausal females are excluded, a highly significant increase in intrinsic clearance in females over males occurs. ($p < 0.001$).

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by

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THESIS

Presented to the Graduate School of Nursing Faculty of

the Uniformed Services University of the Health Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE DEGREE

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

May 1997

FOREWORD

This work is supported by the collaborative efforts of the Graduate School of Nursing and the Department of Pharmacology of the Uniformed Services University of the Health Sciences. In the autumn of 1995, Dr. E. Jane McCarthy and I discussed my potential interests for thesis topics. She learned that I was interested in a purely experimental design for my masters research requirement in nurse anesthesia. This prompted her to introduce me to Dr. Louis Cantilena, chair of the Department of the Clinical Pharmacology at USUHS. I explained to Dr. Cantilena that as a nurse in critical care, I had been often curious about the variability of the sedative effect of the drug Midazolam on the patients I cared for in the ICU. I had an idea that it could be related to gender. Dr. Cantilena suggested that a study could be designed that would explore this question. He opened the doors of his laboratory to me and introduced me a remarkable partner, Aleksandrs Odinecs, a scientist dedicated to the advancement of knowledge in the field of pharmacology. Through long and diligent hours spent together in the laboratory, this work went from idea to reality.

DEDICATION

To the Lord Jesus Christ, and to the most important people in my life, I dedicate the creation of this thesis. Without their love, encouragement, and support, the attainment of a dream and the creation of this thesis would not have been possible.

To my mother, Sylvia G. Humphrey, and father, Richard L. Humphrey, I dedicate this paper and thank them both for instilling within me a strong work ethic and the desire for knowledge and for their many years of encouragement and support.

ACKNOWLEDGEMENT

The assistance, guidance and support of numerous people have contributed to making it possible for me to attain this degree. I am especially grateful to Dr. E. Jane McCarthy, Chairperson, and the members of the Thesis Advisory Committee, Dr. W.Patrick Monaghan, Dr. Eugene Levine, Dr. Louis Cantilena, and Dr. Aleksandrs Odinecs. Their guidance, knowledge and support were invaluable. I am especially indebted to Dr. Odinecs, who gave countless hours of his personal time freely and steered a novice through the shark-infested waters of experimental research.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Research Topic and Problem

There are many factors that can influence how drugs affect the human body. Gender is one factor often overlooked in drug research. When women are the subjects of research, the results are often confounding because the physical and biochemical characteristics of females differ from males. This may mean that they metabolize drugs differently.

Midazolam is a highly metabolized drug possibly influenced by gender. This *in vitro* study, through an experimental approach, looks at the influence of gender on the metabolism of Midazolam (MDZ), the second most frequently administered anesthetic agent in America today, according to a 1996 survey conducted by the Council on Certification of Nurse Anesthetists Professional Practice Analysis.

Background and Significance of the Problem

Midazolam (MDZ) is a benzodiazepine derivative widely administered by nurses in anesthesia and critical care. Its rapid onset and short half life relative to Diazepam make it extremely useful in anesthesia for preoperative sedation, as

an induction agent, or as an adjunct to balanced anesthesia technique. It is frequently chosen by nurse anesthetists as a key agent used for sedating their patients during outpatient diagnostic procedures, or as an adjunct to general or regional anesthesia. An attractive feature of MDZ is the anterograde amnesia it provides. Ensuring amnesia for difficult or unpleasant procedures performed on patients is a primary reason nurse anesthetists choose to administer this drug. Other properties of MDZ useful in anesthesia include skeletal muscle relaxation, blunting of the stress response to surgical stimulation without suppressing adrenocorticoid synthesis, decrease of total peripheral resistance without negative inotropism, and anticonvulsant effects (Lauven & Kulka, 1990; Dundee, Haliday, Harper & Brogden, 1984).

A major frustration for nurse anesthetists who use MDZ to sedate their patients is determining the proper dose for each individual they treat. Short of guesswork, the only solution for the nurse is to administer a reasonable dose, watch for effect, and make adjustments accordingly. Even when such caution is exercised, MDZ can have a variable and unpredictable effect for an unidentified subset of the population. The half life (t 1/2) of MDZ is reported to be 1.5 to 3 hours (Lauven & Kulka, 1990; Allonen, Ziegler & Klotz, 1981). Ninety percent of an oral dose of MDZ is

excreted by the kidney within 24 hours (Heizmann & Ziegler, 1981). In a study of over 200 patients receiving MDZ for induction of anesthesia (0.3 mg/kg), the t 1/2 was markedly prolonged in 6 percent of the patients, ranging from 8 to 22 hours (Dundee, Collier, Carlisle & Harper, 1986).

When it is used during outpatient diagnostic procedures, the patient requires vigilant nursing observation throughout his hospital stay. Centrally mediated respiratory depression, as evidenced by a blunted response to elevated levels of carbon dioxide, does occur with MDZ and can lead to apnea at doses well within the therapeutic range in some persons (Dundee et al., 1984). MDZ's amnestic effect can persist even when the sedation has worn off, prompting nurse anesthetists to instruct their patients not to drive for 24 hours after receiving the drug (Lown et al., 1995). A friend or family member is often required to transport the patient home from the hospital.

The relationship between the level of consciousness and MDZ's serum concentration is highly variable (Oldenhof, de Jong, Steenhoek & Janknegt, 1987). While some persons are unduly sedated, for others, the drug's effect is so mild that the dose must be increased substantially for the desired effect to occur. Repeated doses or continuous IV infusions of the drug can accumulate in the body tissues, causing prolonged sedative effects (Bauer et al., 1995).

MDZ's variability of effect has the potential to dangerously complicate patient care if unwitnessed respiratory arrest occurs. Likewise, an undesirable patient outcome will occur if inadequate dosing results in a lack of amnesia during surgery. At present, there is no way to predict who will be more or less sensitive to the drug's effects. Careful titration of MDZ is a useful technique, but not always effective as a means to deal with this problem. A need exists to characterize those persons who have idiosyncratic responses to MDZ in order to eliminate the guesswork as to what dose is required in a given patient.

There are numerous factors that influence how a given dose of any drug will affect the individual receiving it. These include state of health or disease, blood flow to major organs, the drug's pharmacodynamic profile, ratio of lean body mass to adipose tissue, drug-drug interactions, nutritional background, age, genotype and gender (Katzung, 1995). Deciphering the influence of all these factors is a daunting task. This study begins to unravel a small piece of the puzzle, and in so doing, contribute to the nursing knowledge base concerning the effect of one factor: gender, on the rate of MDZ metabolism.

Historically, the influence of gender has been given little attention in drug metabolism studies, even though it has been known for decades that sex hormones influence the

metabolism of certain drugs (Wilson, 1984). To circumvent this phenomenon, studies were designed using an all-male cohort or by grouping males and females into a homologous whole. Currently, there is an atmosphere of increased awareness of women's health issues and studies are being designed in order to discover what is unique to women's health. To date, although MDZ has been extensively studied, there exists no published *in vitro* data comparing the metabolism of MDZ in humans by gender.

For a drug metabolism study to yield meaningful results, the drug selected must be highly metabolized. Such is the case with MDZ. Less than 1 percent of the drug is excreted unchanged in the urine (Dundee et al., 1984). The biotransformation of MDZ occurs due to the action of enzymes of the cytochrome P450 subfamily, CYP3A. Coincidentally, this subfamily of isozymes is also responsible for the metabolism of endogenous sex steroids in both males and females. MDZ's widespread usage, its high rate of metabolism, and the relationship it shares with sex steroids as substrates for the same enzyme subfamily make it a likely choice as a probe to learn about gender differences in the rate of drug metabolism.

Nursing and Military Relevance

Learning more about the pharmacokinetics of MDZ, one of

the most frequently used drugs in anesthesia today, will advance the practice of the military nurse and nurse anesthetist worldwide. The paucity of scientific research that seeks to define gender-based differences in metabolism is a compelling reason in itself to do this study. It is hoped that this work, sponsored as it is by the U. S. Army, can further the military's goal of providing state-of-the-art health care for its female servicemembers.

Pharmacodymanics of the Drug

MDZ is a 1,4 benzodiazepine derivative with an imidazole ring in the 1,2 position (Fig 1). It was discovered by R. I. Fryer and A. Walser in 1976. MDZ has a unique capacity among drugs in its class to change its lipid solubility based on pH (Smith, Eadie & O'Rourke-Brophy, 1981). At pH values of 4 and below, the imidazole ring opens between the 4 and 5 carbon positions, producing a water-soluble amine derivative. The drug is manufactured and is available for injection at a pH of 3; thus, it is water soluble. Its intravenous administration causes less phlebitis than Diazepam. As it circulates and is exposed to physiologic pH, the imidazole ring closes, making the drug lipid soluble (Gerecke, 1983). MDZ is roughly 5 to 6 times more potent than Diazepam (Mould et al., 1995), with a shorter half life of 1.5 to 3 hours (Allonen et al., 1981;

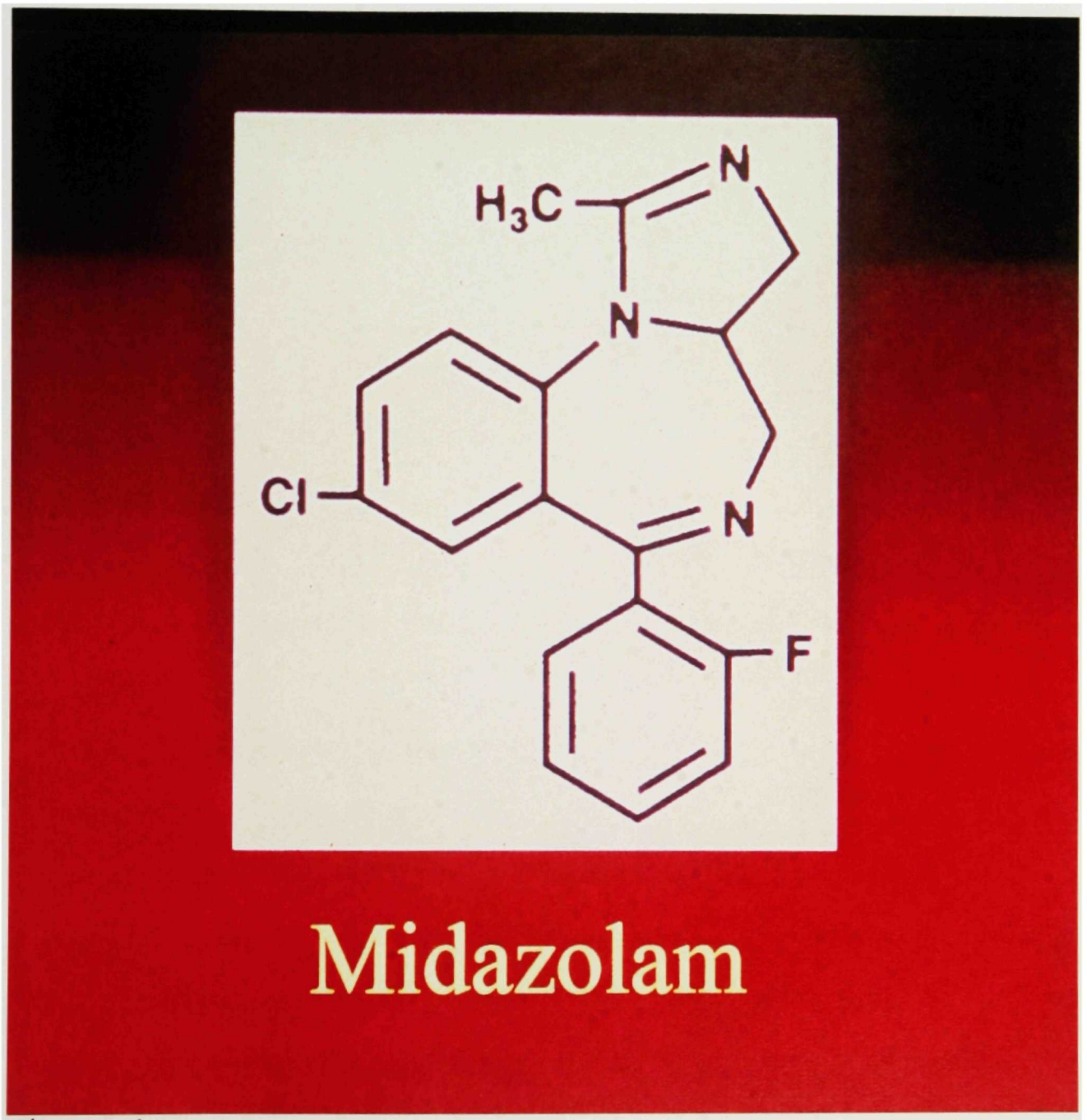

Figure 1.
Chemical Structure of Midazolam

Lauven & Kulka, 1990). MDZ has a rapid onset of action of 3 to 5 minutes when given intravenously (Dundee et al., 1984). Its duration of action is the shortest of any of the benzodiazepines (Gerecke, 1983). This drug provides cardiovascular stability with a slight decrease in systemic vascular resistance, which is attributable to its sympatholytic properties (Dundee et al., 1984). This effect is most pronounced in persons who are hypertensive, dehydrated, or vasoconstricted due to high sympathetic tone. MDZ blocks the catecholamine response to surgical stress, but does not inhibit the renin or cortisol responses to stress (Dundee et al., 1984; Nilsson, 1990). MDZ reduces blood flow to the kidneys, causing decreased glomerular filtration without affecting renal vascular resistance (Calvo, Suarez, Rodriguez-Sasiain & Martinez, 1992). Blood flow to the liver is also reduced, but unless liver disease is severe, this effect does not alter the drug's metabolism (Robin, Lee, Hasan & Wood, 1993). MDZ causes central respiratory depression, as evidenced by a blunted response to elevated carbon dioxide. Title volume is decreased, but respiratory rate increases, resulting in no net change in minute ventilation. Loss of effective function of the abdominal musculature may result in respiratory compromise in persons with chronic obstructive pulmonary disease (Dundee et al., 1984). Dosages of MDZ used for induction of

anesthesia can cause apnea. In some persons, apnea can occur in unexpectedly low doses. Cardiopulmonary arrest has been known to occur and resuscitative equipment should always be readily available. Use of the drug Flumazenil, a benzodiazepine reversal agent, is helpful if the patient has persistent stupor.

Free, unbound MDZ crosses the blood-brain barrier to bind allosterically to GABA receptors in the limbic and subcortical levels of the brain, resulting in sedation (Calvo et al., 1984). Repeated or large doses can produce somnolence and stupor that can last for days (Byatt, Lewis, Dawling & Cochrane, 1984). Other drugs that depress the central nervous system, such as narcotics or alcohol, can potentiate the sedative effect of MDZ (Physicians Desk Reference, 1994).

Pharmacokinetics of the Drug

Oral MDZ is absorbed by the small intestine and delivered to the liver via the portal system before entering the systemic circulation. This circulatory pathway permits the liver to exert its "first pass" effects on MDZ's detoxification before exposing the body to it. The amount of drug that undergoes biotransformation during its first pass through the liver is known as the extraction ratio and is mathematically expressed as:

ER = liver clearance/blood flow to the liver

For MDZ, a high extraction ratio leaves a bioavailability of only 44 percent after the first pass effect is achieved (Allonen, Ziegler & Klotz, 1981). If MDZ is given orally, some of the moderately high extraction ratio is due to the presence of metabolic enzymes in the lumen of the small intestine, which metabolize MDZ even before it reaches the liver (Paine et al, 1996).

The distribution of a drug to the various body organs and tissues is unique for every drug and based on the chemical composition and characteristics of the drug molecule as well as the dose given. MDZ has a volume of distribution (Vd) of 0.44 to 0.91 L/Kg (Allonen et al., 1981). The elimination kinetics of any drug is related to the total plasma clearance (CL) and the Vd by the following formula:

$$t\ 1/2 = 0.7\ Vd/CL$$

Therefore, the half life is increased by an increase in Vd or a decrease in CL. MDZ is cleared almost exclusively by metabolism of the enzyme system cytochrome P450, the majority of which resides in the liver but is also present in the lumen of the small intestine, the kidney, and the

lungs to some extent (Paine et al., 1996; Schuetz et al, 1992; Thummel, O'Shea et al, 1996; Watkins, 1992).

MDZ is approximately 95% bound to plasma proteins, which keeps it from diffusing into the lipid tissues (Lauven et al., 1990). It is the unbound MDZ that is pharmaceutically active. Factors that decrease the amount of circulating plasma proteins or other substances that compete for available binding sites on plasma proteins cause more unbound MDZ to circulate. Even small changes in protein binding can increase dramatically the drug's bioavailability (Calvo et al., 1984). Excessive dosages of MDZ can saturate the proteins, causing the drug to accumulate in the tissues.

The Vd is increased in females, elderly, and obese persons due to a relatively higher percentage of adipose tissue (Dundee et al., 1984). Disease states that increase the extracellular fluid volume can increase the Vd, causing prolonged sedation with MDZ (Malcrida, Fritz, Suter & Crevoisier, 1991; Wandel, Bocker, Bohrer, Browne, Rugheimer & Martin, 1994). The kidney is the organ primarily responsible for eliminating substances from the body by filtration of the blood. The liver, small intestine and, to a lesser extent, the lungs also function to eliminate certain drugs. A drug cannot be filtered by the kidney unless it is in a water-soluble, unbound form. The liver

converts lipophilic organic molecules, such as MDZ, to polar metabolites by biotransformation. These metabolites are then readily filtered and eliminated in the urine. MDZ's hepatic clearance ratio is 30% to 70%, making its metabolic clearance dependent on both liver and enzyme activity and hepatic blood supply (Burtin, Jacqz-Aigrain, Girard, Lenclen, Magny & Betremieux, 1994; Wagner, 1993).

Some researchers argue that the prolonged t 1/2 of MDZ is not due to impaired metabolism but is reflective of an increase in Vd, as seen in elderly, obese, and critically-ill persons (Malicrida et al., 1991; Shafer, Doze & White, 1990; Wandel et al., 1994; Wills, Khoo, Soni & Patel, 1990). Nonetheless, variations in the rate of MDZ metabolism are currently accepted to be the primary mechanism (Allonen et al., 1981; Dundee et al., 1984; Smith et al., 1981).

Metabolic processes can be altered by environmental factors, such as systemic infection (Monshouwer et al, 1995), dietary intake (Kupferschmidt, Ha, Ziegler, Meier & Kruhenbuhl, 1995), or concomitant administration of drugs that competitively inhibit or induce liver enzymes (Lown et al., 1995); Backman, Aranko, Himberg & Olkkola, 1994; Olkkola et al,1993; Pichard et al, 1990; Baldwin et al,1995).

Once biotransformation occurs, the metabolic waste

products are eliminated by the kidney. Clearance of a drug by the kidney is defined as:

CL = Renal elimination of drug/plasma drug concentration

For most therapeutic doses of drugs, the rate of elimination is directly proportional to the drug concentration; that is, the more drug given, the faster it is eliminated. MDZ has a clearance of 5.8 to 9.0 ml/min/kg in a healthy adult (Dundee et al., 1984). A decrease in circulating plasma proteins is symptomatic of kidney failure. This decrease causes the bioavailability of MDZ to increase in kidney failure (Calvo et al., 1992).

MDZ is converted by hydroxylation to three known metabolites: 1'-hydroxymidazolam, 4-hydroxymidazolam, and 1',4-dihydroxymidazolam. This third metabolite does not occur often because its precursor 1'-hydroxymidazolam is usually rapidly conjugated before it can occur. 1'-Hydroxymidazolam is mildly bioactive with a half life of approximately 1 hour. 4-Hydroxymidazolam is inert. The urine output after an ingested dose of MDZ contains 60% to 70% 1'-hydroxymidazolam, 3% 4-hydroxymidazolam, 1% 1'-4-dihydroxymidazolam, and less than 1% unchanged MDZ. Approximately 15% to 25% of the dose is as yet unidentified

(Heizmann & Ziegler, 1981; Smith et al., 1981).

Metabolism is carried out on a cellular level by enzymes that reside in the endoplasmic reticulum of specialized cells, such as are found in the liver. These enzymes are collectively known as the microsomal mixed function oxidase system (MFOS), or cytochrome P450. It is known that the amount of P450 naturally expressed in individuals can vary by up to 2 magnitudes (Guengerich & Turvey, 1991; Thummel, Shen et al., 1994). This means that some persons are able to metabolize drugs more efficiently than others. Since between 5.6% to 6.5% of the population are recognized to be slow metabolizers of MDZ, it would be valuable to able to characterize this group in order to identify susceptible individuals before administering MDZ (Dundee et al., 1986; Wills et al., 1990). At present, little is known about the descriptive characteristics of this group.

Statement of the Problem

There needs to be more information defining the characteristics of rapid and slow metabolizers of MDZ. Specifically, the influence of gender on the rate of MDZ metabolism needs to be identified. There are some studies that indicate that endogenous sex steroids influence the metabolism of MDZ. This may have something to do with the

fact that the same subfamily of cytochrome P450 that metabolizes MDZ (CYP3A) is also responsible for the metabolism of these sex steroids (Gonzales, 1989; Waxman, Attisano, Guengerich & Lapenson, 1988; Wandel et al., 1994; Schuetz et al., 1992; Maenpaa, Pelkonen, Cresteil & Rane, 1993). Endogenous and synthetic hormones can induce or inhibit P450 enzymes (Maenpaa et al., 1993; Schuetz et al., 1992; Kharasch & Thummel, 1993; Wandel et al., 1994; Pichard et al., 1990). Gender-related differences in metabolic rate have been demonstrated for other drugs metabolized by P450 (Lemmens, Burm, Hennis, Gladines & Bovill, 1990; Vanakoski, Stromberg & Seppala, 1993; Kharasch & Thummel, 1993). It is unknown whether P450 metabolism of MDZ is affected by gender, although differences in metabolic rate by gender do occur in rats (Kronbach, Mathys, Umeno, Gonzalez & Meyer, 1989).

Hypothesis

The purpose of this study is to measure the effect of gender on the rate of MDZ metabolism in human liver microsomes to see if differences do indeed exist. This study postulates the following null hypothesis:

Ho: In humans, there is no difference in the rate of metabolism in females versus males.

Definition of Terms

Biotransformation - the conversion of xenobiotics from one form to another within an organism associated with a change in pharmacologic activity.

Half Life (t 1/2) - The time (in hours) required for the drug concentration in the body to decrease by 50%.

High Performance Liquid Chromatography (HPLC) - The separation of chemical substances by differential movement through a stationary and mobile (two-phase) system. The material to be separated is injected through a column of a chosen absorbent; the substances least absorbed emerge the soonest; those more strongly absorbed emerge later.

Intrinsic Clearance - The preferred clearance value used in drug metabolism studies because it is the most sensitive

indicator of metabolic rate. It is calculated by dividing Vmax by Km.

Km - The dissociation constant for Michaelis-Menten kinetics, describing the concentration of a drug at which the velocity of metabolite formation is 50% of Vmax, that is, the enzymes are 50% saturated with substrate. Each type of substrate/enzyme complex has a specific Km value which reflects the affinity of a given enzyme for its substrate. A low Km value means a high affinity and a high Km means a low affinity for the substrate.

Metabolism - The sum of the chemical changes occurring in living tissue, including anabolism and catabolism, and the biotransformation of xenobiotics.

Microsomes - Small spherical vesicles obtained from the endoplasmic reticulum after disruption of cells by ultracentrifugation.

V max - The maximal velocity for a given enzyme in the biotransformation of a specific xenobiotic, ie., the maximal elimination capacity.

Volume of Distribution (Vd) - The amount of drug present in

the body divided by the concentration of the drug in the plasma expressed in liters.

Xenobiotic - A pharmacologically active substance not endogenously produced and therefore foreign to an organism.

Chapter 2

Framework of the Study

The rate of biotransformation by the liver is the most important determinant in the variability of clinical responses seen among individuals to MDZ. This is because MDZ is so highly metabolized. The liver has a myriad of functions, one of which is to detoxify foreign substances, called xenobiotics. Drugs are a form of xenobiotics, as are environmental pollutants, chemicals, and foodstuffs. This metabolic biotransformation is accomplished in the liver by the microsomal mixed-function oxidase system (MFOS). This system includes two enzymatic proteins embedded in the smooth endoplasmic reticulum of hepatocytes: NADPH cytochrome P450 reductase, which is a flavoprotein, and cytochrome P450, a heme protein with a prosthetic group attached. By varying the type of prosthetic group, a number of distinct cytochrome P450's have evolved, each with a specificity for a certain xenobiotic substrate. Cytochrome P450 is so named because when it was first discovered by Klingenberg and Garfinkle in 1958 it was noted to absorb light at 450nm in its reduced form.

Cytochrome P450 is ubiquitous in nature. Over 200 different P450 enzymes have been discovered in mammals, plants, fungi, and bacteria, and new ones are being

discovered all the time. It is thought that the first of the genes that code for these enzymes developed evolutionally about 3.5 billion years ago to equip the eukaryotic cell to leave the watery environment and live on land. Indeed, the oldest known cytochromes are those responsible for synthesizing cholesterol, a crucial constituent of cell membranes (Gonzalez, 1989). There are some 12 families of cytochromes common to all mammals; of these 12 families, 17 subfamilies of cytochromes have been identified in humans so far. Within those 17 subfamilies exist the various individual cytochromes, which are isozymes of each other. The cytochrome classification system is based on the sequence of amino acids in the protein (Nelson et al, 1993). To belong in the same family, cytochromes must be at least 40% similar in their sequence homology. To be in the same subfamily, they must exhibit at least 55% similarity to one another (Gonzalez, 1989; Wandel et al., 1994). The subfamily of cytochromes that metabolize MDZ in humans is known as CYP3A.

Hepatic metabolic biotransformation occurs in two phases. Phase I reactions are mediated by MFOS. A lipophilic compound, such as MDZ, is hydroxylated by a P450 cytochrome. The hydroxyl group then acts as a substrate for transferase enzymes to attach sulfate or glucuronic acid. This attachment is called conjugation and comprises Phase II

reactions. The purpose of the oxidation and conjugation process is to convert lipophilic compounds to polar compounds for renal excretion. Conjugated molecules are water soluble.

The genes that produce the cytochrome P450 enzymes can be stimulated or "induced" to produce more enzyme in response to repeated presence of a xenobiotic. Certain drugs are known enzyme inducers. A person whose liver enzymes are "induced" metabolizes the offending agent more rapidly. An example of a drug that induces CYP3A is Rifampin. Administration of Rifampin to individuals causes them to metabolize MDZ at a dramatically faster rate. The t 1/2 of MDZ in these induced persons drops from 3.1 to 0.2 hours (Backman, Olkkola & Neuvonen, 1996). Benzodiazepines do not induce liver enzymes, but because different cytochrome P450's have distinct yet overlapping specificities, other enzyme-inducing drugs can increase the metabolism of benzodiazepines (Kharasch & Thummel, 1993).

Conversely, other drugs may act as inhibitors of cytochrome by binding with it irreversibly or by decreasing the rate of enzyme expression by the body (Pichard et al., 1989). Erythromycin is an inhibitor of CYP3A. When MDZ is given to someone who has also ingested Erythromycin, the effect of MDZ is greatly increased and the t 1/2 is greatly prolonged (Backman et al., 1994; Lown et al., 1995; Olkkola

et al., 1993). CYP3A is the largest constituent of the total cytochrome P450 content of the human liver, comprising at least 25 percent (Hunt, Westerkam & Stave, 1992). The list of drugs metabolized by CYP3A includes Nifedipine, Verapamil, Terfenidine, Diltiazem, Midazolam, Lidocaine, Cyclosporin, Tamoxoxifen, Lovastatin, Quinidine, Alfentanyl, Progesterone, 17 Alpha-Ethylestradiol, Testosterone, Erythromycin, Carbamazepine, and Phenytoin (Gonzalez, 1989; Kharasch & Thummel, 1993). The variability in CYP3A expression is partially due to enzyme induction, and also due to the wide, inherent genotypic variation in the quantity of cytochromes in different livers, which can vary by up to 2 magnitudes (Thummel et al., 1994; Guengerich & Turvey, 1990).

In humans, the subfamily of CYP3A consists of four isozymes. The isozymes CYP3A3 and CYP3A4 are 98% similar and indistinguishable as far as their behavior is concerned. For this reason, they are often grouped together as CYP3A3/4. CYP3A4 is the most abundant cytochrome present in the uninduced human liver (Gorski, Hall, Jones, VandenBranden & Wrighton, 1994). CYP3A5 is 90% similar to CYP3A3/4, but is only found in 10% to 20% of the population (Gonzalez, 1989). CYP3A7 is the predominant cytochrome expressed in fetal liver and is not found elsewhere (Bertin et al, 1994).

CYP3A3/4 is responsible for the majority of MDZ biotransformation to 1'-Hydroxymidazolam in most individuals. In the 10% to 20% of the population where CYP3A5 is expressed, it exhibits a different reaction specificity, compared to CYP3A3/4 (Wandel et al., 1994). For example, Erythromycin, Quinidine and Cyclosporine are poorly metabolized by CYP3A5, compared to CYP3A3/4. In contrast, Nifedipine and Midazolam make excellent substrates for CYP3A5 (Thummel et al., 1994).

CYP3A5 metabolizes MDZ 2 to 3 times more rapidly than CYP3A3/4. It is eight times more likely to convert MDZ to the 1'-Hydroxy form over the 4-Hydroxy form of the metabolite. The amount of 1'-Hydroxymidazolam produced has been used as a predictor of CYP3A5 content (Gorski & Hall et al., 1994).

In addition to the liver, three alternate sites of cytochrome P450 activity exist: the kidney, small intestine, and the lungs. CYP3A5 occurs primarily in the kidney (Wandel et al., 1994; Schuetz et al., 1992). This secondary site of metabolism, in addition to the small intestine, could help explain why liver disease does not compromise MDZ metabolism (Robin et al., 1993). Currently, investigators are pursuing the possibility that the lungs could play a role in the metabolism of MDZ by CYPB1, a cytochrome that has some similarities to CYP3A (Wandel et al., 1994).

Overall, the determining factors for site of metabolism are the blood flow to the specific organ and the amount of cytochrome P450 in that organ, which establishes the liver as the predominant site for the metabolism of MDZ (Wandel et al., 1994).

As an aside, Fabre and his colleagues first suggested that MDZ was metabolized by CYP3A in 1988 (Fabre et al, 1988). Kronbach et al (1989) identified MDZ as specifically metabolized by CYP3A4. Wandel et al (1994) demonstrated that MDZ was also metabolized by CYP3A5, which is coded for by a separate gene in roughly 20% of the population. By using concentrations of MDZ in excess of the therapeutic level in man, Gorski et al (1994) were able to predict the presence of CYP3A5. This was possible because of the higher ratio of 1'-Hydroxy to 4-Hydroxymidazolam formation unique to CYP3A5 (Gorski et al., 1994). Thummel et al (1994) did an in vivo study using liver transplant recipients to measure total CYP3A content as it related to MDZ clearance. MDZ clearance was found to be highly correlated with total CYP3A content, as measured from liver biopsy specimens obtained within 24 hours of drug administration (Thummel et al., 1994). For this reason, MDZ is considered an excellent probe to measure CYP3A activity in man.

Chapter 3

Review of the Literature

It is well known that the disposition of drugs in humans is under the influence of several gender-related factors. The proportion of adipose tissue relative to lean muscle mass is greater in females than in males, causing females to have a greater Vd and thus a greater distribution of drug and slower drug clearance. Sex hormone binding globulin levels are 1.5 times higher in women than in men, which influences the percentage of bioactive drug in the body circulation for highly protein-bound drugs, like MDZ. Numerous physical changes occur as the result of the menstrual cycle in women, including fluctuations in water and electrolyte levels and surges in estradiol, follicle stimulating hormone (FSH) and progesterone. Mid-cycle, during ovulation, increases in the metabolic clearance of some drugs have been described. Use of oral contraceptives abolishes the mid-cycle hormonal surge and likewise the increase in drug clearance (Wilson, 1984).

In mammals, the hypothalamic-pituitary axis regulates the secretion of the sex steroids. Hypothalamic releasing factors act on the pituitary gland, causing it to secrete follicle stimulating hormone and luteinizing hormone. These hormones, in turn, act directly on the gonads, stimulating

their production of sex steroids. The circulating levels of sex steroids then act as a negative feedback mechanism on the hypothalamic-pituitary axis.

Evidence supporting the effect that the hypothalamic-pituitary axis has on drug metabolism does not exist in humans, yet in the rat model much is understood. In rats, sex steroids act indirectly on the liver through their feedback effect on the pituitary gland. This effect differs by gender. At birth, testicular androgens irreversibly program the brain of rats relating to its influence on hepatic metabolism of drugs. In this way, male rats metabolize MDZ faster than females (Kronbach, 1989). This effect seems to be the reverse of what has been elucidated so far in human studies (Kronbach, 1989).

In humans, data support the conclusion that the effect of age on the pharmacokinetics of CYP3A substrates is gender-dependent. Greenblatt et al (1984), did an <u>in vivo</u> study comparing the total clearance of MDZ in adult males versus females by age They found that males tend to clear MDZ less efficiently with increasing age, but not so with females. Using human liver microsomes, Hunt compared CYP3A metabolic activity in males versus females using Erythromycin as a probe. She found that females had on average 24% more CYP3A activity than males, regardless of age, smoking history, alcohol use, or body weight (Hunt et

al., 1992). Watkins et al, in 1989, developed a tool called the Erythromycin Breath Test in which a small dose of radioactive Erythromycin was ingested and the product of the metabolism, radioactive carbon 14 was measured by having the patient exhale into a small receptacle. Erythromycin, being a specific substrate for CYP3A, was used to indirectly measure CYP3A activity in this way. Watkins found that women had approximately 30% more carbon 14 than males.

Preliminary data using human liver microsomes to study the metabolism of Nelfinavir, a new antiviral CYP3A substrate, demonstrated that females metabolize the drug two times faster than males (Wu et al, 1996). Thummel et al have extensively studied MDZ as a CYP3A probe for several years. In a 1994 study where livers of donors for transplant recipients were used, it was found that the livers of seven female donors had 2 times the CYP3A content of the 14 male donors (Thummel et al,1994). In Thummel et als most recent study (1996), they found no gender-related differences in either oral or IV MDZ clearance in vivo using a sample of ten males and ten females. The study did not indicate the subject's age or fertility status. The study also included <u>in vitro</u> work, comparing MDZ metabolism in liver versus small intestine, but not by gender.

In reviewing the literature, it is found that the data concerning the influence sex hormones play in the clearance

of CYP3A substrates are confusing. The endogenous sex hormones estrogen, progesterone and testosterone are known to be metabolized by CYP3A (Gonzalez, 1989; Kharasch et al., 1993; Pichard et al., 1990). Testosterone is also metabolized by CYP2C8 and CYP4B1 (Mauenpaa et al., 1993). Mauenpaa and his colleagues demonstrated that MDZ strongly inhibits the hydroxilation of testosterone in human liver microsomes. Bocker et al (1991) found that oral contraceptives inhibit CYP3A activity. There is an irreversible binding and destruction of CYP3A when it complexes with norethisterone, ethylestradiol (Ortiz de Montellano & Kunze, 1980), and the synthetic progestin Gestodene (Guengerich, 1990).

In summary, although some data exist that hint of a relationship between gender and rate of CYP3A substrate metabolism, the mechanism is not apparent. This is probably due to a lack of research using females that discriminates by age and fertility status. In addition, the data are conflicting in in vivo studies versus in vitro studies. This may be due to the ability of in vitro studies to isolate one or two variables not possible in a living person.

Chapter 4

Methodology

Overview

The experimental design described in the ensuing chapter is one which is well established in pharmacology research. Prior to the experiment, a number of steps were required to prepare for the actual study, including assay development, selection of an internal standard, and derivation of calibration curves, followed by a pilot study.

In this experiment, human liver microsomes, the isolated enzyme extract that catalyzes the chemical reactions specific to the body's detoxification, are used under controlled laboratory conditions to measure the hydroxylation of MDZ. Each microsome sample has the explicit and unique characteristics of the human donor from which it came. Hence, the ability exists to exclude all extraneous variables from the phenomenon under consideration; the enzyme velocity in MDZ metabolism, male versus female.

However, the liver being removed from the human body and apart from all the influences of that realm and its surrounding environment, causes this design to be an artificial construct. Therefore, one cannot directly extrapolate the results of this study or any similar study

to the living world of humans at large. This study serves as a clue to one of many interconnected factors influencing drug metabolism and disposition.

The liver microsomes used in this study were provided under the auspices of the Washington Regional Transplant Consortium, Washington, D. C., an agency whose task it is to facilitate the deposition of living human tissue used in transplantation and research. All of the persons whose liver tissue was used in this study met sudden and untimely death. Either they or their family spokesperson elected to make the gift of organ donation. Although these livers were subsequently found to be unsuitable for transplantation, they were welcome and fit for use in scientific research, which in itself may contribute to the welfare of society. In conclusion, this study would not have been possible without the generous and ultimate gift of living tissue from these human beings.

For obvious reasons, the identity of the donors is kept anonymous, but certain salient, descriptive data accompany each liver sample to the receiving laboratory. Table 1 describes the characteristics of the twelve donors, five male and seven female, used in this study.

Microsome Preparation

Microsomes were prepared from the human liver samples found unsuitable for transplantation. The livers are transported from the hospital to the laboratory on ice generally within three hours of harvesting and, upon arrival, are sliced into cubes in a bath of cold Eurocollins transplantation buffer solution. The liver cubes are packed into 50 ml plastic tubes and stored at -80 °C. until such time as they are needed to be made into microsomes. To prepare the microsomes, the liver cubes are thawed in a buffer solution and are subjected to ultracentrifugation to separate the microsomal fraction from the rest of the sample. The microsomal pellet is then suspended in a buffer solution of 0.1 uM phosphate buffer containing 1 mM EDTA and 50 uM MgCl2, pH 7.4. Once the microsomes are prepared in this manner they are frozen at -80 °C until needed for experimental purposes.

Each liver, and therefore each microsome, have a unique protein concentration. It was therefore necessary to standardize this protein concentration in all microsomes used for this study. To do this, the protein content of each microsome was assessed using the Bio-Rad Protein Assay, BioRad Laboratories (Richmond, CA). The protein content of the microsomes was then adjusted to yield a standard concentration of 0.2 mg protein/ml.

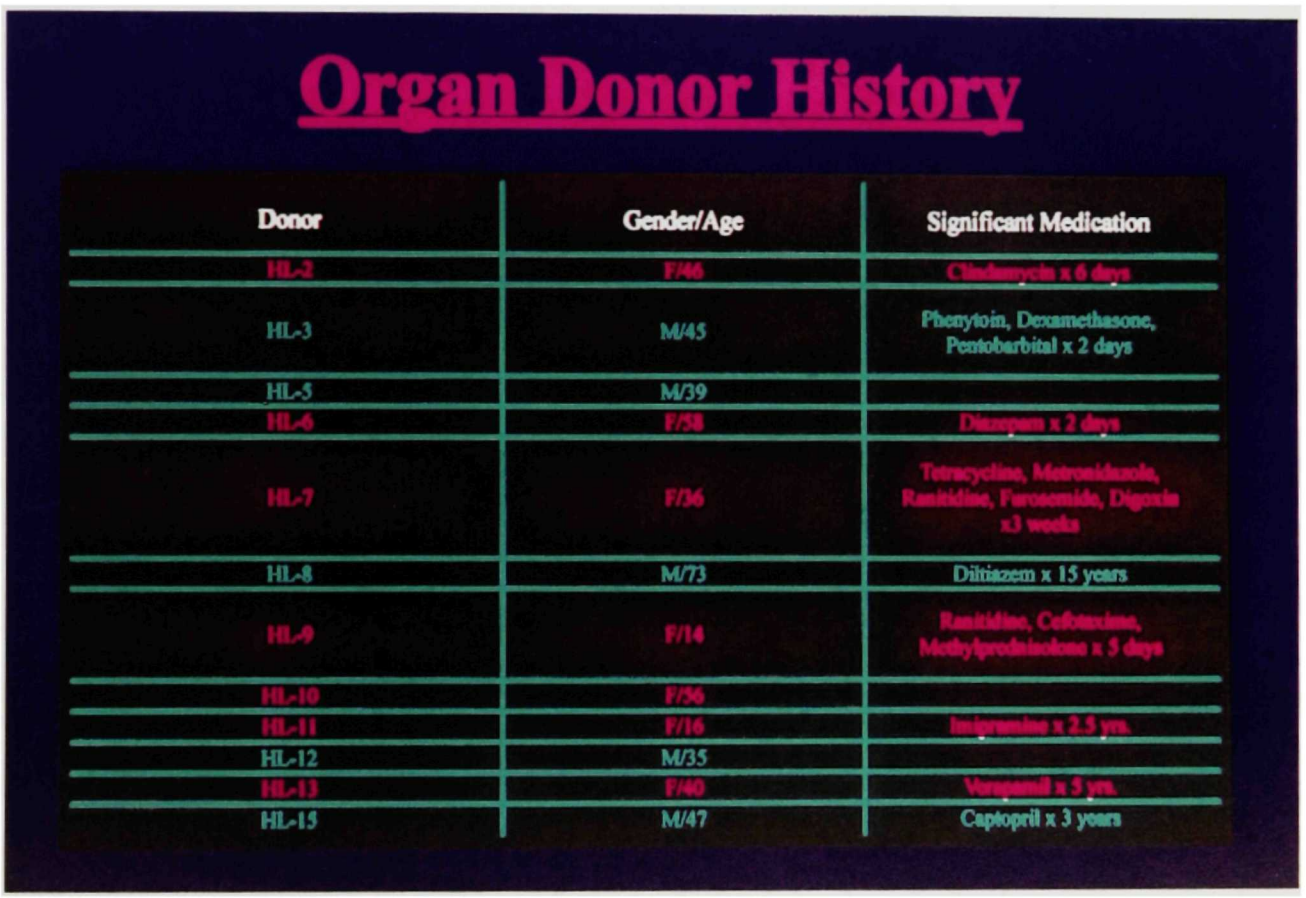
Organ Donor History
Donor
Gender/Age
Significant Medication
HL-2
F/46
Clindamycin x 6 days
HL-3
M/45
Phenytoin, Dexamethasone, Pentobarbital x 2 days
HL-5
M/39
HL-6
F/58
Diazepam x 2 days
HL-7
F/36
Tetracycline, Metronidazole, Ranitidine, Furosemide, Digoxin x3 weeks
HL-8
M/73
Diltiazem x 15 years
HL-9
F/14
Ranitidine, Cefotaxime, Methylprednisolone x 5 days
HL-10
F/56
HL-11
F/16
Imipramine x 2.5 yrs.
HL-12
M/35
HL-13
F/40
Verapamil x 5 yrs.
HL-15
M/47
Captopril x 3 years

Table 1.

Assay Development

In order to study the effect of MDZ on human microsomes, an accurate and sensitive measuring tool was required. Mass spectrometry has been used successfully for this purpose, but it is highly sophisticated and expensive equipment not available to most laboratories. The goal was to develop an assay that was simple in its design, replicable at any reasonably equipped laboratory suited for this purpose, and sensitive enough to yield meaningful data using drug concentrations as close as possible to those found in clinical conditions in vivo.

With this purpose in mind, the Hewlett-Packard High-Performance Liquid Chromatography (HPLC) System, model 1050, was used and an original assay was developed for it to measure MDZ. Through multiple trial and error, the various components of this system were selected and adjusted to yield optimum results. Different absorbent columns, which filter the drug and its metabolites, were tried until the Prodigy 5 micron ODS (3) 100 angstrom column by Phenomenex Company (Torrence, CA) was selected because of its ability to provide the best separation of the analytes. The column temperature was thermostatically controlled at 35° C. The HPLC system was refitted with microbore tubing to permit the use of smaller quantities and slower flow of eluent. The mobile phase solution is acetonitrile: sterile water

(90:10), selected for its ability to dissolve a variety of relatively insoluble compounds.

Because MDZ is pH sensitive, and in order to get sharp peaks with good separation between peaks, the pH of the acetonitrile had to be decreased slightly. Through experimentation, incremental quantities of formic acid was titrated to the acetonitrile until the optimal concentration of acetonitrile with 0.05% formic acid was selected. This concentration gave the solution a pH of just over 4.0 when it was tested with reagent. Altering this pH in either direction caused the peaks to elute at different retention times, widen, or merge together. The mobile phase pump speed was set at 0.2 ml/minute. The injection volume was 15 uL, and the run time was 60 minutes. The lamp wavelength, which gave the best visibility, was adjusted to 220 nm. The flow rate gradient (time in min:flow rate in ml) was 0:0.2, 2:0.2, 3.5:0.25, 5:0.25, 30:0.25, 32:0.25, 33:0.4, 39.8:0.4, 40:0.25, 45:0.25, 55:0.2 and 60:0.2. The gradient conditions (time in min:% formic acid in acetonitrile) were 0:85, 5:85, 30:55, 32:2, 40:2, 45:15, and 60:15.

This assay was proven accurate for detection of MDZ and its metabolites at concentrations ranging from 40 to 0.5 micromolar, at the injection volume of 15 uL. Slightly less sensitivity is expected using biological media (microsomes) because some of the drug will bind to the proteins and be

lost in the extraction process. However, after the experiment was completed, the calculated sensitivity was 32 ng/ml (0.1 micromolar). The physiologic concentration of MDZ in an average adult after a 1 mg IV dose is somewhere around 30 to 50 ng/ml, maximum. With larger doses, the concentration increases.

Selection of Internal Standard

Once MDZ and its two metabolites were clearly visualized on the HPLC system with a well-developed assay, the next process involved selection of an appropriate internal standard. The ideal internal standard is a drug close in chemical composition to MDZ so as to be detected by the MDZ assay, yet not so similar as to be confused with MDZ or its two metabolites. This means the drug must have a different retention time than either MDZ or its metabolites on the HPLC system and also be clearly identifiable in this assay. Experimentation with several other benzodiazapines led to the selection of Lorazepam at 31 uM concentration because it eluted as a tall, sharp peak at 29.1 minutes' retention time. MDZ (24.7 min.), alphahydroxymidazolam (27.1 min.) and 4-hydroxymidazolam (22.9 min.) have distinctly different retention times, yet not so dissimilar as to be incomparable. Use of an internal standard is important because during the extraction portion of the

experiment, some of the drug and its metabolites are inadvertently wasted and are unrecoverable. By adding a known quantity of internal standard and calculating the ratio of internal standard peak-height to MDZ and metabolite peak-height, it is possible to overcome any inconsistencies that would have resulted from basing the results on peak-height of MDZ and its metabolites alone. Using this peak-height ratio ensures accuracy of the data. This ratio is then compared to the actual known concentration of the drugs obtained from the construction of calibration curves. In this way, meaningful data about the actual quantity of metabolites formed during the incubation phase of the experiment are obtained.

Chemicals and Reagents

Midazolam, alphahydroxymidazolam, and 4-hydroxymidazolam were gifts from F. Hoffman-LaRoche, A. G. (Basel, Switzerland). The internal standard Lorazepam was provided by Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). Constituents of the NADPH generating system were purchased from Sigma Chemical Co. (St. Louis, MO).

Standard Solutions

Standard stock solutions of MDZ and its metabolites were prepared at a concentration of 3.2 mM in ethanol. Dilutions of the 3.2 mM standards were used to make the appropriate working solutions of MDZ and its metabolites. A standard stock solution of Lorazepam was prepared at 3.11 mM in ethanol and further diluted to prepare the working solution at 5 uM. Stock and working solutions were stored at -10° C. for the duration of the study, which lasted three months.

Calibration Curves

A mixture of equal parts of MDZ and its two metabolites was prepared from the stock solutions and diluted with sterile water until the resulting concentration was 100 uM. This solution was then serially diluted with sterile water to yield standards at concentrations ranging from 80 to 5 uM. A standardized microsome suspension was then inoculated with each of the above-mentioned concentrations and 200 uL of Lorazepam (31 uM), then extracted per standard procedure. Calibration curves were generated by least-square regression analysis of the analyte/internal standard peak-height ratio versus the concentration of the analyte.

Precision and Accuracy

Intra-assay precision was assessed from analysis (n = 4) of spiked microsome suspension at two different concentrations (50 uM and 25 uM). The microsome suspension was also spiked with 200 uL of internal standard stock solution. Recovery was assessed by comparing the peak-height ratios of the analyte/ internal standard in two sets of extracts.

Pilot Study

After all the components and tools for the study were assembled and made ready to use, it was necessary to test the experimental plan. To this end, one microsome sample was utilized and the entire experiment was carried out exactly as it would be done. In this manner, the feasibility of the process was tested and small adjustments to the procedure were made as a result of the data obtained.

Experiment

The experimental design consisted of four broad phases: Preparation of the incubation media (microsomes), timed incubation of the MDZ/media mixture, extraction of the metabolites, and finally, HPLC analysis. For each of the twelve microsome samples, seven different concentrations of MDZ were used: 50, 100, 200, 400, 600, 800, and 1200 uM. In

addition, all samples of each concentration were performed in triplicate. This yielded 252 samples for analysis, plus controls and standards.

Preparation of Incubation Media.

The microsomes, having been stored in liquid nitrogen, were thawed and diluted by phosphate buffer to yield a final protein concentration of 0.2 mg protein/ml of incubation medium. Each microsomal suspension was warmed in a shaking water bath at 37° C. for three minutes after adding 10 uL of an NADPH generating system, which provided the energy needed to fuel the reaction. The NADPH generating system was prepared by mixing 846 mg glucose-6-phosphate, 252 mg NADP+, 2460 uL buffer, 540 uL glucose-6-phosphate-dehydrogenase, to total 3,000 uL, enough for 300 samples.

Incubation Phase

Ten uL of the 7 concentrations of MDZ were added at 0 minute and the reaction was incubated at 37° C. for 5 minutes, then stopped by plunging the test tubes into wet cubed ice. Two hundred uL of Lorazepam (5 uM) was added to each sample and standards after the tubes were in ice.

Extraction Process

Five ml of acetonitrile was added to the incubation medium and the tubes were vortexed for ten minutes. The tubes were centrifuged at 2,000g and 5° C for ten minutes. All incubation media were then transferred to clean tubes and labeled. The tubes were evaporated to dryness with a speed vacuum apparatus. Twenty uL of acetonitrile: water (1:1 v/v) were added and the tubes vortexed for three minutes. Two ml of acetonitrile were added to each tube and the tubes were again centrifuged at 2,000g and 5 C. for ten minutes. The contents of the tubes were transferred to clean tubes and labeled. The tubes were again evaporated to dryness with a speed vacuum apparatus. Following this, 20 uL of acetonitrile:water (1:1 v/v) were added to each tube and the tubes were vortexed for two minutes. Again, 20 uL of water were added to each tube and the tubes were vortexed for two minutes. Each sample was then transferred to a microvial and loaded onto the HPLC system.

HPLC Analysis

Each of the samples required an hour to analyze on the HPLC system. The output from the system consists of both graphic and tabular data. The graph depicts the retention times of MDZ, its metabolites, and Lorazepam. These are depicted on the graph by means of a tall peak rising

from a flat baseline. The clarity of the tall, sharply defined peaks rising from a flat baseline, as shown in Fig. 1, are characteristic of a well designed and executed assay.

The height of each peak was measured from base to tip by the computer, as well as the time in minutes that the peak occurred during the hour analysis. Each of these computer-generated values was verified and if found erroneous were corrected by hand in a process called integration. The raw data were then ready for entering into the Excel spread sheet data base for analysis.

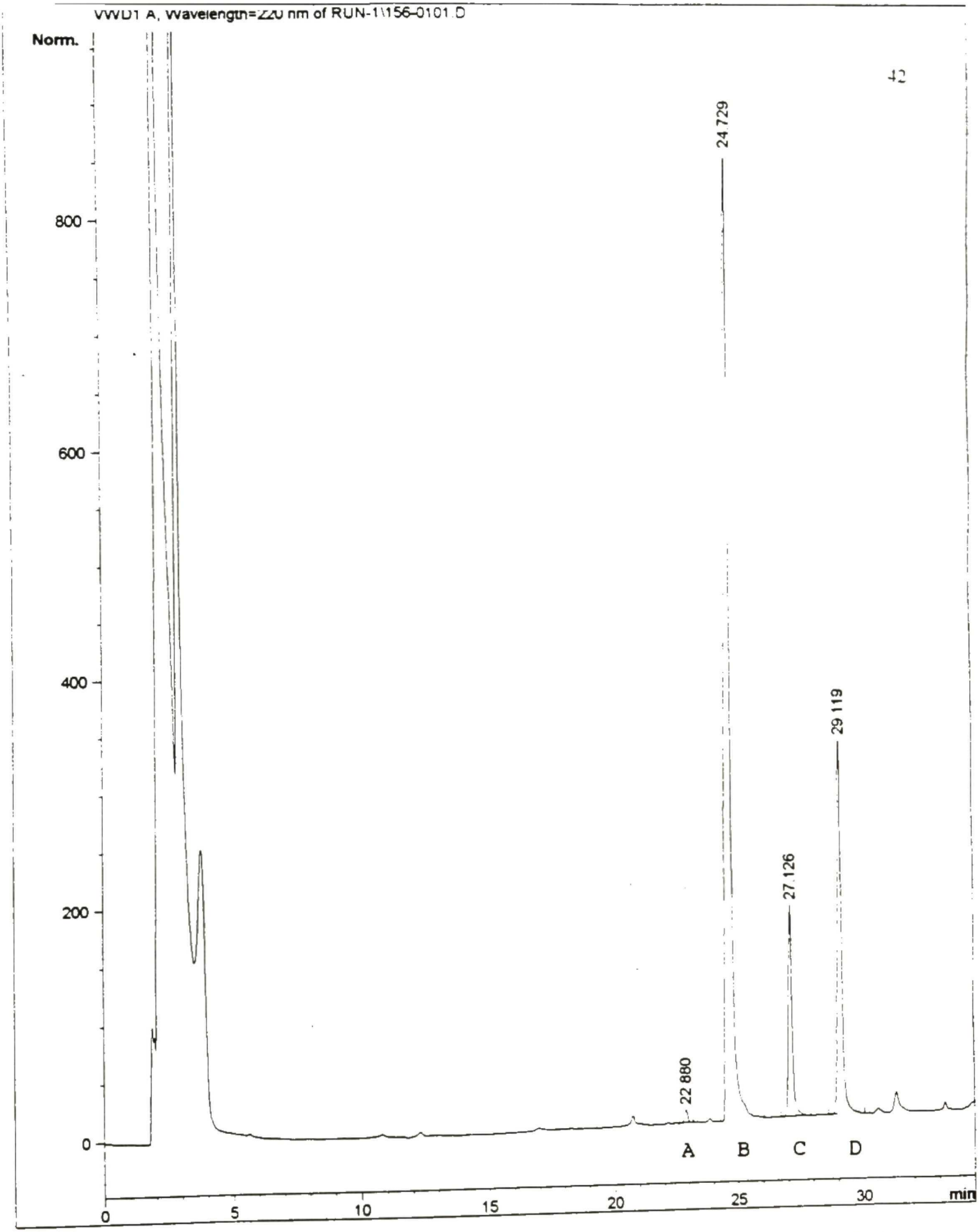

FIGURE 2. Sample chromatogram showing 4-hydroxymidazolam (A), MDZ (B), 1'-hydroxymidazolam (C), and Lorazepam (D).

Chapter 5

Data Analysis

Analytical Method

A total of 252 samples (12 x 3 x 7) were analyzed, excluding calibration samples and controls (See Appendix). This data consisted of tall peaks on a graphic scale occurring at fixed time intervals unique for each metabolite and the parent compound. The internal standard Lorazepam was also observed at its fixed time interval (Figure 1). The height of the peaks of MDZ and its metabolites were divided by the peak height of the internal standard to derive a ratio. From this data, the presence of MDZ and its metabolites were visually detectable but not quantifiable. For this reason, additional calibration samples, made up of several known concentrations of MDZ, its two metabolites, and Lorazepam were compiled. When these samples were analyzed, the height of each peak could be graphed against the known concentration of the compound. The points derived were used to construct a line. A weighting scheme (1/y) was used to protect the lowest points on the line. A ten times higher weight was given to the lowest point than was given to the highest point on the line. The y intercept and the slope of this line were calculated and the equation of the line derived according to this formula:

$$y = a + bx$$

y = peak height of drug/internal standard peak height

x = actual drug concentration in uM

a = y intercept

b = coefficient

Once these calibration curves were established, the uM concentration of the experimental data set could be calculated algebraically by substituting y in the equation with the peak heights of the experimental data. Grubbs' test for statistical outliers was applied to the data to exclude extraneous points. The mean and standard deviation of the data were calculated and a 95% confidence interval was chosen before any further analysis was conducted.

Km and Vmax values for the 1' and 4 hydroxylation of MDZ were estimated for each microsomal sample by nonlinear regression analysis using the software package PCNONLIN (Scientific Consulting, Inc., Apex, NC). The regression analysis measures the averages between points and generates a curve that follows the average path. An example of the curve generated by the subject "HL-5" for the velocity of 1-hydroxy metabolite formation is shown in Figure 3. The intrinsic clearance (CLint) for each microsomal sample was calculated as the ratio of Vmax/Km. Correlation analysis was done using the StatView software package (Abacus

Concepts, Inc., Berkeley, CA).

The formation of 1'- and 4-hydroxymidazolam expressed as Vmax and intrinsic clearance values were highly correlated with each other ($r = 0.35$, $p = 0.34$). This is to be expected for metabolites formed by the same enzyme. The data obtained for the formation of 4-hydroxymidazolam were felt to be potentially unreliable. Because the 4-hydroxy metabolite makes up only about 4% of an administered dose of MDZ, these data were excluded from further analysis and the major metabolite, 1'-hydroxymidazolam, was focused upon (Tables 2 and 3).

There was extreme individual variability among the samples in the velocity of substrate metabolism. Calculated as a ratio between the maximal and minimal values, the catalytic activity of 1'-midazolam hydroxylase varied 38 fold by Vmax, 5 fold by Km, and 8 fold by Clint (Table 4). Maximal variability in velocity for 1'-hydroxymidazolam formation was obtained for the second highest concentration used, 8.0 uM, which varied 24 times. This was in contrast with the 1.0 uM concentration, which varied only 10 times (Table 4). The increase in variability of 1'-midazolam hydroxylation was statistically significant ($r = 0.98$, $p = 0.0006$).

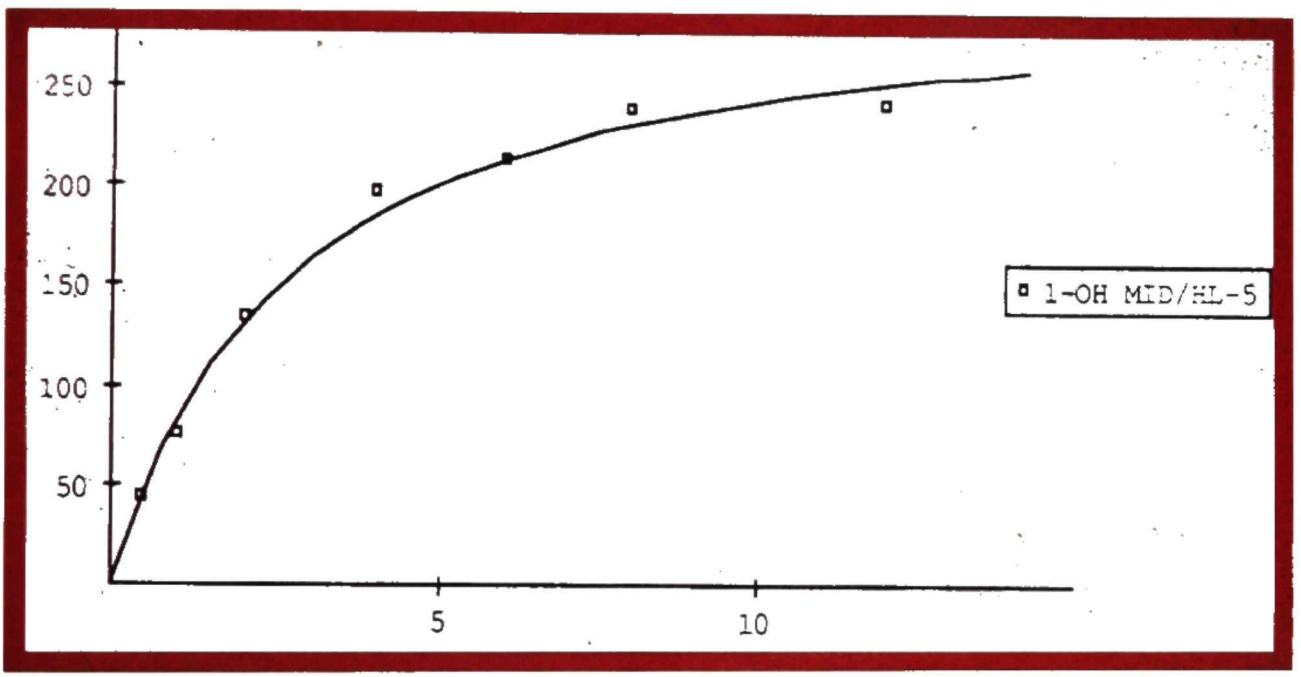

Figure 3.
Representitive curve depicting the velocity of
1'-hydroxymidazolam formation in a single test sample

Results of the Test of the Hypotheses

The hypothesis of this study states that in humans, there is no difference in the rate of MDZ metabolism between males and females. Analyzed as a whole, differences by gender in the intrinsic clearance values from MDZ biotransformation to 1'-hydroxymidazolam were not statistically significant, ($p = 0.06$), according to the T test of means. However, when postmenopausal females were excluded from the data, the difference in intrinsic clearance values became highly significant (396 vs. 136 uL/min/mg protein; $p = 0.02$). Similarly, 1'-hydroxymidazolam Vmax and Km values were not significantly higher in females than males until postmenopausal females were excluded (Tables 2and 3, and Figures 4,5,6).

A noticeable increase in the female/male ratio of 1'-hydroxymidazolam formation occurred with a increase in substrate concentration (Table 4). The velocity of MDZ hydroxylation at a concentration of 1.0 uM was 2.6 times higher, and at a concentration of 8.0 uM was 3.6 times higher.The mean Vmax value was 4.3 times higher in females than in males. This phenomenon was statistically significant ($r = 0.96$, $p < 0.006$).

1'-Hydroxymidazolam Formation (pmol/min/mg microsomal protein) after Incubation of Midazolam concentrations

Donor	0.5 uM	1.0 uM	2.0 uM	4.0 uM	6.0 uM	8.0 uM
HL-2	221	420	820	1369	1906	2210
HL-3	95	148	241	382	499	528
HL-5	44	75	134	198	214	239
HL-6	N.D.	77	131	176	206	245
HL-7	N.D.	424	737	1126	1415	1786
HL-8	102.5	183	329	422	558	679
HL-9	N.D.	461	829	1426	2144	2648
HL-10	N.D.	145	286	437	596	677
HL-11	56	97	163	293	290	309
HL-12	27	44	79	82	105	110
HL-13	102	236	427	668	815	771
HL-15	43	67	130	121	161	171

mean values from 3 separate samples

Table 2.

TABLE 3. Estimated Michaelis-Menten parameters for the formation of 1'-hydroxymidazolam by human liver microsomes

Gender	Vmax (pmol/min/mg protein)	Km (uM)	CLint (uL/min)
males:			
HL-3	872	4.95	176.2
HL-5	309	2.65	116.6
HL-8	1043	4.94	211.1
HL-12	138	1.99	69.3
HL-15	215	1.98	108.6
Mean +/- SD	515 +/- 413	3.3 +/- 1.52	138.4 +/- 58.6
females:			
HL-2 (1)	4085	7.52	543.2
HL-6 (2)	336	3.39	99.1
HL-7 (1)	2919	6.01	485.7
HL-9 (1)	5276	9.52	554.2
HL-10 (2)	1066	5.33	200
HL-11 (1)	451	3.14	143.6
HL-13 (1)	1395	5.51	253.2
Mean +/- SD (1,2)	2218 +/- 1915	5.76 +/- 2.25	325.8 +/- 195.8
Mean +/- SD (1)	2825 +/- 1955*	6.24 +/- 2.37*	396.0 +/- 186.3*

1- Female age < 60, 2- Female age > 60, * Values with a statistically significant difference between male and female age groups

Table 3.

Variability and Female/Male Ratio at Different Substrate Concentrations

Substrate (uM)	Variability	Variab. Female	Variab. Male	Vf/Vm
0.5	N.D.	N.D.	N.D.	N.D.
1	10.4	265+/-167	103+/-59	2.57
2	10.5	485+/-307	183+/-101	2.65
4	17.4	785+/-519	241+/-153	3.26
6	20.5	1053+/-776	307+/-207	3.43
8	23.9	1236+/-967	346+/-246	3.57
12	N.D.	1324+/-1024	N.D.	N.D.
Vmax	38.2	2218+/-1915	515+/-413	4.31

Table 4.

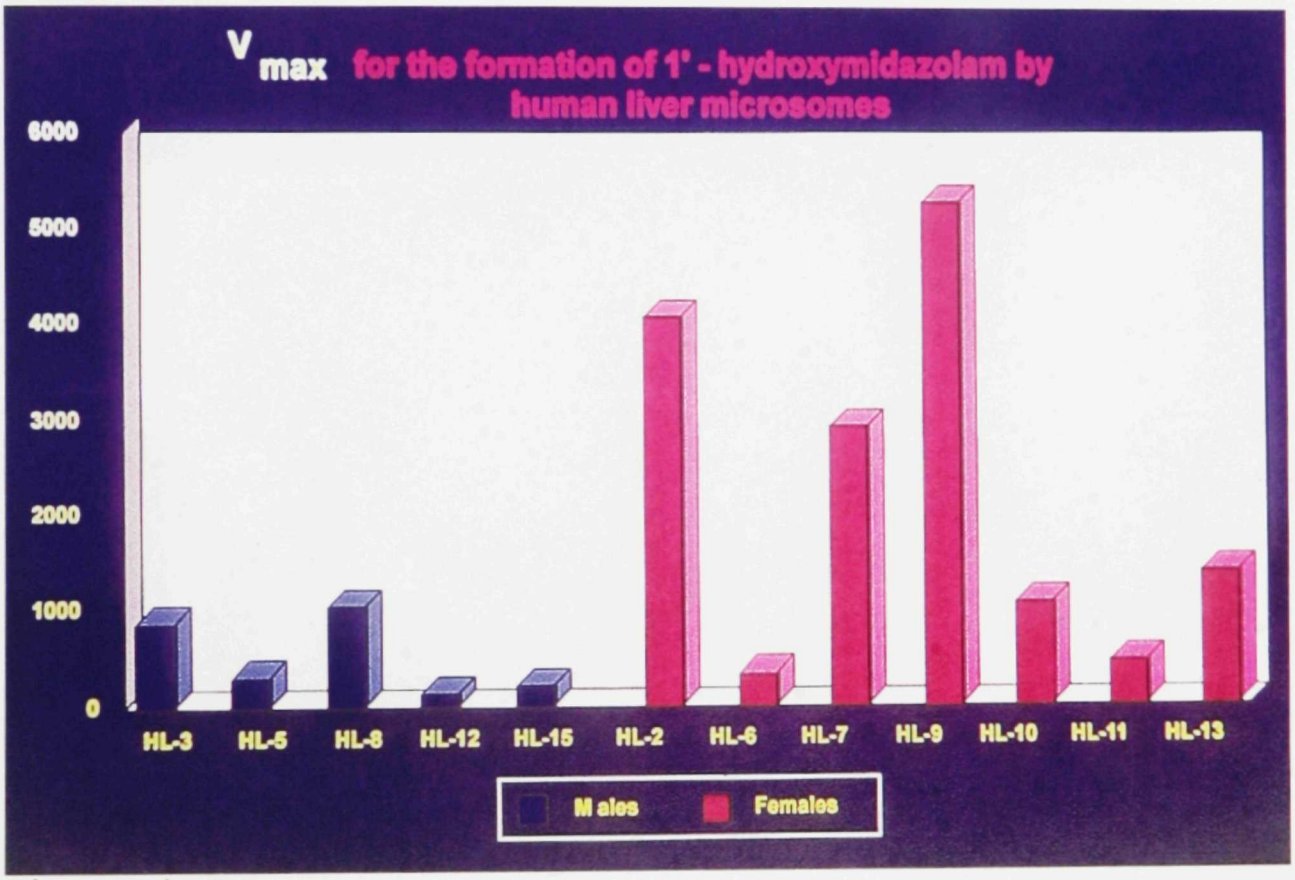
V max for the formation of 1' - hydroxymidazolam by human liver microsomes
6000
5000
4000
3000
2000
1000
0
HL-3
HL-5
HL-8
HL-12
HL-15
HL-2
HL-6
HL-7
HL-9
HL-10
HL-11
HL-13
Males
Females

Figure 4.

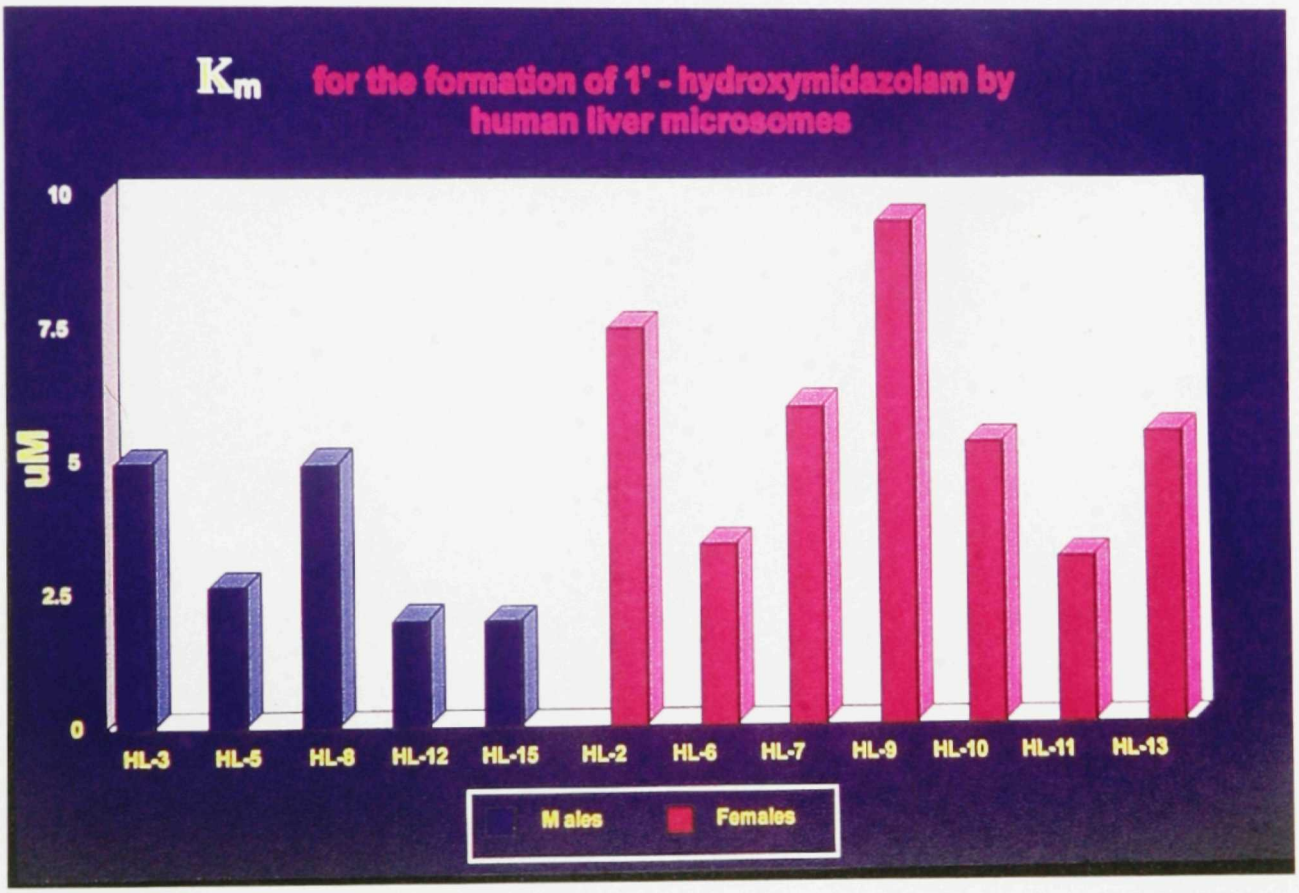
Km for the formation of 1' - hydroxymidazolam by human liver microsomes
10
7.5
5
2.5
0
uM
HL-3
HL-5
HL-8
HL-12
HL-15
HL-2
HL-6
HL-7
HL-9
HL-10
HL-11
HL-13
Males
Females

Figure 5.

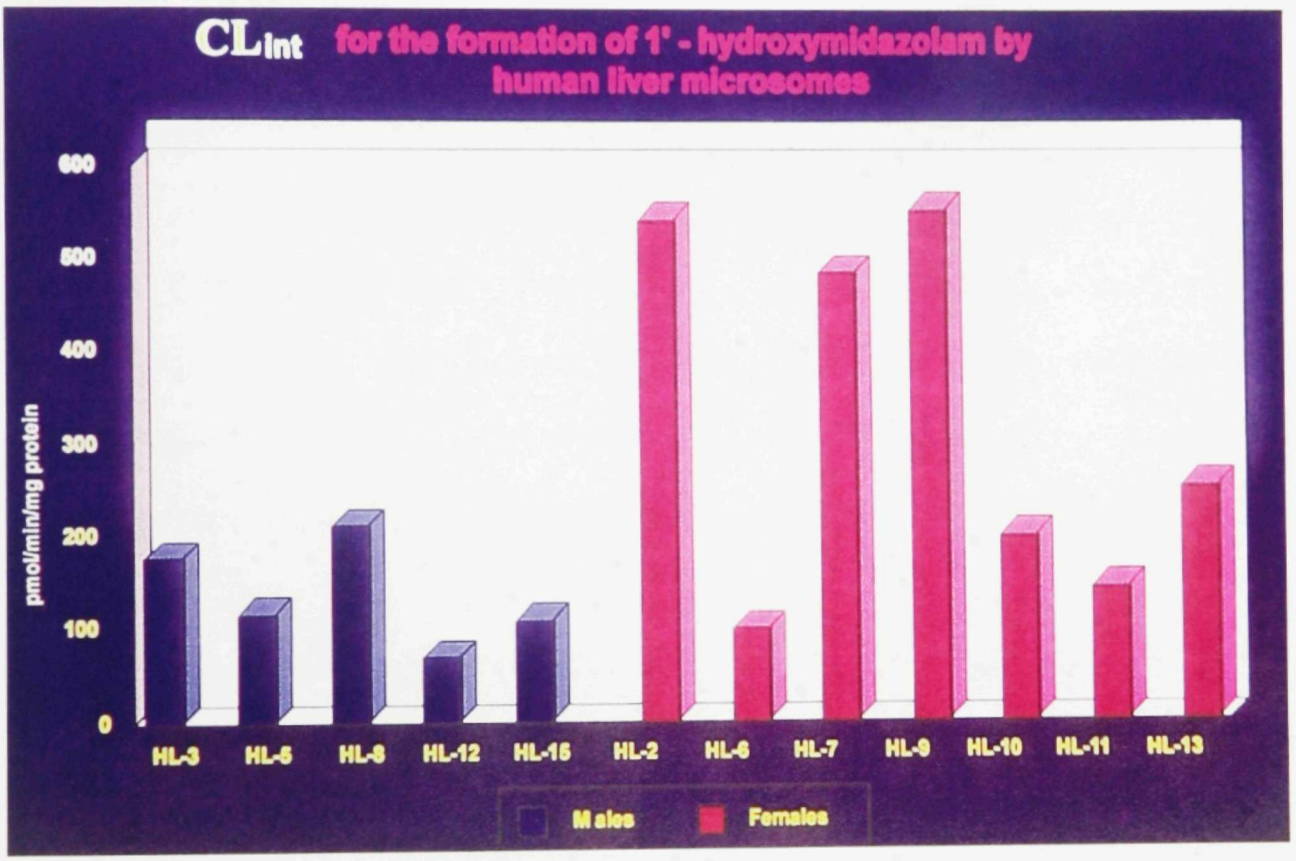
CLint for the formation of 1' - hydroxymidazolam by human liver microsomes
pmol/min/mg protein
0
100
200
300
400
500
600
HL-3
HL-5
HL-8
HL-12
HL-15
HL-2
HL-6
HL-7
HL-9
HL-10
HL-11
HL-13
Males
Females

Figure 6.

Chapter 6

Discussion and Conclusions

In previously published studies to date, no attempt has been made to evaluate the effect of gender on the metabolism of MDZ in vitro. Therefore, these data are unique and at present a comparative analysis to other previously published studies cannot be done.

Analysis of the medical history of the donors, as presented in Table 1, shows that only one person (HL-3, M/45) received what are considered by Pichard et al (1990) to be CYP3A inducers, Phenytoin, Dexamethasone, and Pentobarbital. The exact dosing regime or time frame required to induce someone's liver enzymes with a given drug has never been accurately defined and probably varies by individual. Since this person only received these medications over a 48-hour time interval, he was not eliminated from the male group for gender differences evaluation.

The data obtained in this study describing the in vitro metabolism of MDZ in human liver microsomes are in the same range as recorded previously (Kronbach et al., 1989; Gascon & Dayer, 1991; Ha, Rentsch, Kneer & Vonderschmitt, 1993; Thummel et al., 1996). Wandel et al (1994) reported much higher values for the Km of 1'-hydroxymidazolam formation.

A reasonable explanation would be that they selected, as a lowest concentration, one which is quite higher than that usually selected for Km analysis.

The results of this study disprove the null hypothesis, in that the metabolic capacity of females of reproductive age to degrade MDZ to 1'-hydroxymidazolam was significantly higher than in males. For 1'-hydroxymidazolam formation the Km value was 2 fold higher in females. At higher concentrations of substrate, gender differences became even more pronounced. Analysis of female/male ratios of 1'-hydroxymidazolam formation shows a positive correlation between female/male ratio and substrate concentration. This confirms speculation that in concentrations much lower than Km, gender-related differences are less pronounced than at high concentrations.

The results of this study suggest that CYP3A activity in human liver is higher in females than in males, which is in accord with other published <u>in vitro</u> data showing higher Erythromycin N-demethylation activity, another established probe for CYP3A, in female vs. male liver microsomes (Hunt et al., 1992).

In contrast to Erythromycin, where <u>in vitro</u> gender-related differences were confirmed <u>in vivo</u>, previously published literature for MDZ indicates no statistically significant, gender-related differences in the

pharmacokinetics (Greenblatt et al., 1984; Holazo, Winkler & Patel, 1988). No in vivo differences were observed among women based on age, as well as the use versus nonuse of oral contraceptives (Avram, Fragen & Caldwell, 1983; Greenblatt et al., 1984; Holazo et al., 1988).

Obesity has been observed to have a significant effect on the pharmacokinetics of MDZ, as reported by Greenblatt et al (1984). There are approximately 2 times less total clearance in obese persons (Greenblatt et al., 1984). The discrepancy between in vitro and in vivo data might be explained by the assumption that two factors:1; the high extent of protein binding of MDZ, and 2; body fat content, play a more important role in individual variability in the pharmacokinetics of MDZ than does the rate of metabolic degradation of free MDZ. If this is true, the rate of MDZ elimination is limited by the rate of drug-protein complex dissociation and release from the fat depot, and not by its rate of metabolic degradation. This hypothesis is in accord with the assumption made by Wills et al (1990) that prolonged t 1/2, observed in a small proportion of the population is secondary to an increase in Vd and not a result of alterations in clearance and metabolism. Published data regarding gender differences in the pharmacokinetics of drugs that are CYP3A substrates are controversial. For some of these drugs, such as

Erythromycin (Austin, Mather, Philpot & McDonald, 1980; Kahan et al,1986), Alfentanyl (Lemmens et al., 1990; Rubio & Cox, 1991), Diazepam (Greenblatt, Allen, Harmatz & Shader, 1980), and Methylprednisolone (Lew et al,1993) a significantly higher clearance value in females was reported, implying the faster rate of metabolism by females than males. Conversely, gender differences in the pharmacokinetics of Nifedipine (Lobo, Jack & Kendall, 1986), a second well-recognized probe for CYP3A activity, were not observed. In the case of Nifedipine, as well as MDZ, factors other than CYP3A activity must be playing a key role in intersubject variability of the pharmacokinetics of these drugs.

Common also for MDZ and Nifedipine is that their therapeutic concentrations in vivo are much lower than their Km values. Data from this study demonstrate an increase in formation of 1'-hydroxymidazolam in females of reproductive age over males, corresponding with an increase in substrate concentration. It is, therefore, logical to assume that more pronounced gender differences can be expected for drugs whose physiological concentrations are higher than their Km values.

There are several physiological possibilities to explain the increased rate of metabolism of MDZ in females compared to males. The higher metabolic capacity of women

during their reproductive years might be a mechanism that developed through evolution to protect the unborn child from partial poisoning. As stated earlier, it may also be due to the higher body fat content or differences in protein binding. It can only be speculated as to why these phenomena exist. The role of gender in drug metabolism remains a mysterious and controversial subject to which insufficient time and energy have been devoted. Since it is ethically infeasible to obtain liver samples from live persons, these types of studies are done with donated liver samples from persons who have died and whose health history is largely obscured due to confidentiality issues. It would be interesting but impractical, for example, to do a study using liver samples from females in various stages of their menstrual cycle to see what role hormone fluctuations play in drug metabolism. At the present time, there is no way to do this study in vitro, and in vivo work is confounded by other pharmacokinetic influences, such as protein binding and fat distribution.

This study is important as the first of its kind to demonstrate that females do metabolize MDZ faster than males <u>in vivo</u>. This discovery was uncovered only after extra effort was undertaken to separate the female subjects into pre- and postmenopausal age groups. There is much more research to be done in this area. First, replication

studies using a larger cohort segregated by age and gender are needed to see if these results are reproducible. Further work determining the actual CYP3A isozyme content of the subjects using gel-electrophoresis would enable corelation of CYP3A content with Km and Vmax values per subject, thus providing another clue as to why gender differences do exist. Use of known CYP3A inducers or inhibitors added to the incubation media, such as sex steroids, would be a way to further explore the notion that hormones play an influential role in the gender difference of MDZ metabolism. It is hoped that this study can be part of a foundation for future research in the field of women's health, and ultimately contribute to the safer practice of anesthesia in women.

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